FORM PCT 1390 REV. 5/93 U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NO. PFALLER-1 (PCT) US
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371	U.S. APPLICATION NO. (if known, see 37 CFR 1.5) 10/031547

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP00/06091	JUNE 29, 2000	JULY 22, 1999

TITLE OF INVENTION: PYRF GENE AND ITS USE

APPLICANT(S) FOR DO/EO/US Rupert PFALLER

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. X This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ___ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. X This is an express request to begin national examination procedures (35 U.S.C. 371 (f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. X A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. X A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. X is transmitted herewith (required only if not transmitted by the International Bureau)
   b. ___ has been transmitted by the International Bureau.
   c. ___ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. X A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ___ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
   a. ___ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ___ have been transmitted by the International Bureau.
   c. ___ have not been made; however, the time limit for making such amendments has **NOT** expired.
   d. ___ have not been made and will not be made.
8. ___ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. X An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ___ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ___ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. X An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. X A **FIRST** preliminary amendment.
    ___ A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. ___ A substitute specification.
15. ___ A change of power of attorney and/or address letter.
16. X Other items or information:
    2 Sheets of formal Drawings
    Sequence Listing-Paper Copy-Disk Copy
    Statement Under Rules 821 (e)(f) and (g) and 825 (a) and (b).

Applicant Claims Priority under 35 U.S.C. §119 of Germany Application No. 199 34 408.6 filed July 22, 1999.
Applicant Claims Priority under 35 U.S.C. §120 of: PCT No. PCT/EP00/06091 filed June 29, 2000

531 Rec'd PCT/PT 18 JAN 2002

APPLICATION NO. (if known, see 37 CFR 1.5) 10/031547	INTERNATIONAL APPLICATION NO. PCT/EP00/06091	ATTORNEY'S DOCKET NO. PFALLER-1 (PCT) US

X The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)):		
Search Report has been prepared by the EPO or JPO.....**$890.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) **$710.00**		
Neither international preliminary examination fee paid (37 CFR 1.82) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....**$1,040.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).....$100		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 890.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ___ 20 ___ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).		

Claims	Number Filed	Number Extra	Rate		
Total Claims	9 - 20 =	- 0 -	X **$18.00**	$	
Independent Claims	4 - 3 =	- 1 -	X **$84.00**	$ 84.00	
Multiple dependent claim(s) (if applicable)			+ **$270.00**	$	
TOTAL OF ABOVE CALCULATIONS =				$ 974.00	
Reduction by 1/2 for Small Entity status				$	
SUBTOTAL =				$	
Processing fee of **$130.00** for furnishing the English translation later than ___ 20 ___ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
TOTAL NATIONAL FEE =				$	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$ 40.00	
TOTAL FEES ENCLOSED =				$ 1,014.00	
				Amount to be: refunded	$
				charged	$

___ Applicant claims Small Entity status.

a. X A check in the amount of $ 1,014.00 to cover the above fees is enclosed.

b. ___ Please charge my Deposit Account No. 03-2468 in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. X The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment, to Deposit Account No. 03-2468. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
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Edward Freedman
Signature

Edward R. Freedman
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Express Mail No. EL 871451535
Date of Deposit January 18, 2002

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10, on the date indicated above, and is addressed to Box PCT, U.S. Patent and Trademark Office, P.O. Box 2327, Arlington, VA 22202.

Lisa L. Vulpis
Lisa L. Vulpis

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: PFALLER - 1 (PCT) US

PCT No.: PCT/EP 00/06091 FILED: JUNE 29, 2000

TITLE: PYRF GENE AND ITS USE

PRELIMINARY AMENDMENT

BOX PCT
U.S.P.T.O.
P.O. Box 2327
Arlington, VA 22202

Dear Sir:

Preliminary to Examination, please amend the above-identified application as follows:

IN THE ABSTRACT

Please insert the Abstract attached hereto on its own separate page.

IN THE DRAWINGS

Please add one additional sheet of drawings showing new FIGS. 3, 4, 5 and 6.

IN THE SPECIFICATION

On Page 1, below the title, and above line 3, please insert as follows:

--CROSS REFERENCE TO RELATED APPLICATIONS

Applicant claims priority under 35 U.S.C. §119 of German Application No. 199 34 408.6 filed July 22, 1999. Applicant also claims priority under 35 U.S.C. §120 of PCT/EP00/06091 filed June 29, 2000. The international application under PCT article 21(2) was not published in English.

BACKGROUND OF THE INVENTION

1. Field of the Invention--

On Page 1, in line 7, please insert:

--2. The Prior Art--

On Page 2, in line 20, please insert:

--SUMMARY OF THE INVENTION--

On Page 8, please amend the paragraph in lines 12 to 21 to read as follows:

--DNA sequences which are involved other than as transcription terminators at the 3' end of the protein-encoded gene in the expression and secretion of the expressed gene can likewise be present in the DNA vector of the invention. One example thereof is provided by the gene for the laccase from Neurospora crassa, whose 3' end contains the sequence for 13 amino acids which are deleted during secretion of the protein and are no longer present in the mature protein (Germann et al., J. Biol. Chem. (1988) 263. 885-896).--

On Page 12, please amend the paragraph in lines 20 to 27 to read as follows:

--In a preferred embodiment of the invention, the filamentous fungus Trametes versicolor is transformed in a homologous system with the gene of a laccase from Trametes versicolor. This achieves an increase in the expression rate for said laccase, which significantly improves the production rate in the fermentation of 0.1 g of laccase/l of culture medium which can be achieved in the prior art.--

On Page 15, in line 8, please insert:

--BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the vector pTV gap being 3.7 kb in size;

FIG. 2 shows the clone pPyr Fgap being 5.3 kb in size;

FIG. 3 shows the SEQ ID NO: 4 for Primer A;

FIG. 4 shows the SEQ ID NO: 5 for Primer B;

FIG. 5 shows the SEQ ID NO: 6 for Oligo PyF-1; and

FIG. 6 shows the SEQ ID NO: 7 for Oligo PyF-2.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS--

On Page 16, please amend the paragraph in lines 9 and 10 to read as follows:

--Primer A: (See FIG. 3) SEQ ID NO: 4

Primer B: (See FIG. 4) SEQ ID NO: 5--

On Page 19, please amend the paragraph in lines 1 to 12 to read as follows:

--3.4 kb of sequence information was found from the longest of the pyrF clones. This pyrF clone was called pyrF61 (SEQ ID NO: 1). The pyrF61 clone contained sequence information for the pyrF structural gene (coding region, SEQ ID NO: 1, bp, 1133-1877). The coding sequence region additionally contained an intron (SEQ ID NO: 1, bp 1226-1286) which is not translated into

amino acid sequence. The corresponding pyrF cDNA gene is indicated in SEQ ID NO: 2. The pyrF structural gene present in the pyrF61 clone, without the intron sequence, codes for a protein having the amino acid sequence indicated in SEQ ID NO: 3.--

On Page 20, please amend the paragraph in lines 9 to 12 to read as follows:

--Oligo PyF-1 (See FIG. 5) SEQ ID NO: 6

Oligo PyF-2 (See FIG. 6) SEQ ID NO: 7--.

A Marked-Up Version of Specification Pages 1, 2, 8, 12, 15, 16, 19 and 20 is enclosed.

IN THE CLAIMS:

Please cancel claims 1 to 7 without prejudice, and please replace by inserting new claims 8 to 16, as follows:

8. A DNA sequence which codes for a protein having an enzymatic activity of orotate phosphoribosyl-transferase (pyrF activity) which comprises

a DNA sequence selected from the group consisting of

the DNA sequence SEQ ID NO: 1 in a region from position 1133 up to and including position 1877,

the DNA sequence SEQ ID NO: 2 in a region from position 1 up to and including position 684,

a DNA sequence having a sequence homology of more than 70% with the said region of the DNA sequence SEQ ID NO: 1, and

a DNA sequence having a sequence homology of more than 70% with the said region of the DNA sequence SEQ ID NO: 2.

9. A protein having pyrF activity, which comprises

an amino acid sequence selected from the group consisting of

the amino acid sequence SEQ ID NO: 3; and

an amino acid sequence having a sequence homology of more than 70% with the amino acid sequence SEQ ID NO: 3.

10. An expression vector which comprises a DNA sequence as claimed in claim 8.

11. A microorganism which comprises an expression vector as claimed in claim 10.

12. A process for producing fungal strains which are capable of efficient expression and secretion of proteins, comprising

transforming a fungal strain with an auxotrophic gene defect as host strain in a transformation mixture, using with an expression vector which has a gene for complementation of the auxotrophic gene defect in the host strain;

selecting clones transformed with the expression vector from the transformation mixture by selection for complementation of the auxotrophic gene defect;

controlling expression of the gene for complementation of the auxotrophic gene defect in the host strain by a genetic regulatory element which is active in the host strain; and

employing as host strain a uridine-auxotrophic fungus selected from the group of genera consisting of Trametes, Coriolus and Polyporus with a gene defect in the pyrF gene.

13. An expression system comprising

a host strain selected from the group of genera consisting of Trametes, Corilous and Polyporus having a genetic defect in metabolism, on the basis of which the metabolite uridine which is essential for growth is no longer synthesized, and the host strain is no longer able to grow on minimal media without addition of this metabolite; and

an expression vector comprising a selection marker gene which complements the auxotrophic gene defect of the host strain, wherein the host strain has a genetic defect in metabolism a defect in the pyrF gene, and the selection marker gene is the pyrF gene from a fungus of the class Basidiomycetes.

14. A process for producing a protein, which comprises employing an expression system as claimed in claim 13 comprising a gene encoding the protein in a manner known in a culture for protein production; and

obtaining the protein from the culture.

15. A process for producing a protein, which comprises cultivating in a culture a microorganism as claimed in claim 11, comprising a gene encoding the protein; and

obtaining the protein from the culture.

16. A process for producing a protein, which comprises cultivating in a culture a fungal strain produced by a process as claimed in claim 12, comprising a gene encoding the protein; and

obtaining the protein from the culture.

REMARKS

By this Preliminary Amendment, the Specification has been amended to include a cross-reference to related applicatons which

has been inserted in page 1. Also pages 1, 2 and 15 have been amended to recite the Specification section headings required by U.S. practice. In addition, pages 16 and 20 of the U.S. Specification have been amended to conform to the requirements of U.S. practice as it relates to biotechnology. Thus the nucleotide bases for SEQ ID NOs: 4, 5, 6 and 7 have been canceled from these pages 16 and 20 and now appear in the drawings as new FIGS. 3, 4, 5 and 6, respectively. Furthermore, pages 8, 12 and 19 of the Specification have been amended to conform to the changes made in the International Office.

The amendments to the claims are to cancel the originally filed claims 1 to 7 without prejudice, and to replace these claims with new claims 8 to 16. New claims 8 to 16 eliminate the multiple dependency of the claims, so as to avoid the U.S.P.T.O. surcharge therefor. Also claims 8 to 16 have been written so as to comply with U.S. formal requirements. Claims 8 to 16 correspond to the amended claims filed in the International Office.

An Abstract of the Disclosure on its own separate page is enclosed.

No new matter has been introduced by this amendment. Entry of this amendment is respectfully requested.

Respectfully submitted,

PFALLER - 1 US (PCT)

By: Edward R. Freedman

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Enclosure: (1) Abstract of the Disclosure;
(2) Marked-Up Version of Amended Specification pages 1, 2, 8, 12, 15, 16, 19 and 20;
(3) New Drawing FIGS. 3, 4, 5 and 6.

EXPRESS MAIL # EL 871451535 US
DATE: January 18, 2002

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10, on the date indicated above, and is addressed to the U.S. Patent and Trademark Office, Box PCT, P.O. Box 2327, Arlington, VA 22202.

Lisa L. Vulpis

Lisa L. Vulpis

ABSTRACT OF THE DISCLOSURE

A pyrF gene is useful as a selection marker gene for an expression system for the production of proteins in mushrooms of the genus Trametes, Coriolus or Polyporus. The pyrF gene includes a DNA sequence SEQ ID NO: 1 from position 1133 up to and including position 1877 or DNA-sequence SEQ, ID NO: 2 from position 1 up to and including position 684 or a DNA-sequence with a sequence homology greater than 60% relative to the above-mentioned regions of sequence SEQ ID NO: 1 or SEQ ID NO: 2.

MARKED-UP VERSION

OF

AMENDED SPECIFICATION

pyrF gene and its use

CROSS REFERENCE TO RELATED APPLICATIONS BACKGROUND OF THE INVENTION

1. Field of the Invention

The invention relates to a pyrF gene and its use as selection marker gene for an expression system for producing proteins in fungi of the genera Trametes, Coriolus or Polyporus.

2. The Prior Art

Various prokaryotic and eukaryotic expression systems are known for producing proteins. The application DE-A-19814853 describes in detail the prior art in this regard. DE-A-19814853 itself discloses a process for transforming filamentous fungi from the genera Trametes and Polyporus, with which it is possible to achieve significantly higher production rates for a protein expressed in each case. The application discloses expression vectors which comprise genetic regulatory elements for expression in filamentous fungi of the class Basidiomycetes. On transformation of filamentous fungi of the class Basidiomycetes they permit positive transformants to be selected on the basis of the complementation of an auxotrophic gene defect.

The gene defect disclosed in DE-A-19814853 relates to the pyrG gene. This gene codes for orotidine-5'-phosphate decarboxylase. DE-A-19814853 also discloses strains with a defect in the pyrG gene which are able to grow on minimal medium only in the presence of uridine (uridine auxotrophy). After transformation of these strains with DNA vectors which comprise an intact pyrG gene, the uridine-auxotrophic strains again grow on minimal medium without uridine (uridine prototrophy).

Uridine-auxotrophic strains are isolated in the state of the art (Boeke et al., Methods Enzymol. (1987) 154, 164-175) by treatment with the genotoxic substance 5-fluoroorotic acid (FOA). Uridine-auxotrophic strains generated on treatment with FOA have a genetic defect either in the pyrG gene or in the pyrF gene. The pyrF

gene is also called the ura5 gene. It codes for the enzyme orotate phosphoribosyltransferase.

Uridine-auxotrophic Basidiomycetes strains with a defect in the pyrF gene would also be valuable strains for transformation with the aim of producing proteins if the intact pyrF gene from Basidiomycetes were available as selection marker gene for efficient transformation. However, pyrF genes have to date been described only for fungi from the class Ascomycetes such as, for example, Podospora anserina (Gene 53 (1987), 201-209), Kluyveromyces lactis (unpublished, the DNA sequence is deposited in the "Genbank" database under the accession number klj001358.gb_pl) or Yarrowia lipolytica (M. Sanchez et al., Yeast 11 (1995), 425-433). On the other hand, no pyrF genes from filamentous fungi from the class Basidiomycetes such as, for example, of the genera Trametes, Coriolus or Polyporus are known.

Summary Of The Invention

One object of the present invention is to provide pyrF genes from filamentous fungi from the class Basidiomycetes. These genes are suitable for use as selection marker genes for the transformation of uridine-auxotrophic strains.

The present invention relates to a DNA sequence which codes for a protein having the enzymatic activity of orotate phosphoribosyltransferase (pyrF activity), which comprises the chromosomal DNA sequence SEQ ID NO: 1 from position 1133 up to and including position 1877, or comprises the cDNA sequence SEQ ID NO: 2 from position 1 up to and including position 684, or comprises a DNA sequence having a sequence homology of more than 60% with the DNA sequence SEQ ID NO: 1 or SEQ ID NO: 2.

A preferred DNA sequence has a sequence homology of more than 70% with the DNA sequence SEQ ID NO: 2.

be brought about, for example, by inserting a recognition sequence for a protein-cleaving enzyme into the linkage site between the secretion carrier and the protein to be secreted. An example of this which may be mentioned is the lysine-arginine recognition sequence for the so-called KEX2 protease and an example of a secretion carrier is the glucoamylase from Aspergillus niger (Contreras et al., Bio/Technology (1991) 9, 378-381, Broekhuijsen et al., J. of Biotechnology (1993) 31, 135-145).

DNA sequences which are involved other than as transcription terminators at the 3' end of the protein-encoding gene in the expression and secretion of the expressed ~~expression~~ gene can likewise be present in the DNA vector of the invention. One example thereof is provided by the gene for the laccase from Neurospora crassa, whose 3' end contains the sequence for 13 amino acids which are deleted during secretion of the protein and are no longer present in the mature protein (Germann et al., J. Biol. Chem. (1988) 263, 885-896).

Preparation of the expression vectors of the invention takes place by methods known in the prior art. Various possibilities are explained in the examples. The methods described therein can be applied by the skilled worker to any desired other vectors, resistance genes, regulatory elements and structural genes.

The invention further relates to microorganisms which comprise an expression vector of the invention.

Microorganisms suitable for the expression of an expression vector of the invention are strains of filamentous fungi from the class Basidiomycetes.

Strains from the genera Trametes, Coriolus and Polyporus are particularly suitable.

uridine-auxotrophic filamentous fungus selected from the genera Trametes, Coriolus and Polyporus. The relevant strain from the class Basidiomycetes may be a monokaryotic or else a dikaryotic strain. In a preferred embodiment, it is a uridine-auxotrophic strain which has a defect in the pyrF gene.

Particularly preferred for the transformation is a monokaryotic, uridine-auxotrophic, pyrF-deficient strain from the species Trametes versicolor.

The selection of positive transformants takes place, for example, by placing protoplasts, after transformation with vector DNA, on a medium to which is added, for osmotic stabilization of the protoplasts, an addition such as, for example, sorbitol, mannitol or sucrose and which allows the selection of transformants with the complementing pyrF gene.

In a preferred embodiment of the invention, the filamentous fungus Trametes versicolor is transformed in a homologous system with the gene of a laccase from Trametes versicolor. This achieves an increase in the expression rate for said laccase, which significantly improves the production rate in the fermentation of 0.1 g of laccase/l of culture medium which can be achieved in the prior art.

Preferably used for this purpose is the promoter which is intrinsic to the laccase gene or the promoter for a strongly expressed gene from Trametes versicolor. The promoters of the laccase genes I and III, whose isolation and use is described in DE-A-19814853, are preferably used. The promoter of another strongly expressed gene is represented by the GAPDH promoter for the glyceraldehyde-3-phosphate dehydrogenase from Trametes versicolor.

Selection media preferably used are those on which only

which has been produced by the process of the invention.

Such production processes are known in principle, for example from Eggert et al., Appl. Environ. Microbiol (1996) 62, 1151-1158, Martinez et al., Appl. Microbiol. Biotechnol. (1994) 41, 500-504, or WO 93/08272.

BRIEF DESCRIPTION OF THE DRAWINGS

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The following examples serve to illustrate the invention further. The standard methods used in the examples for treating DNA or RNA, such as treatment with restriction endonucleases, DNA polymerases, reverse transcriptase etc., and the standard methods such as transformation of bacteria, Southern and Northern analysis, DNA sequencing, radiolabeling, screening and PCR technology were, unless indicated otherwise, carried out as recommended by the manufacturer or, if no manufacturer's instructions were available, in accordance with the prior art known from standard textbooks.

1st example

Isolation of a pyrF-specific DNA probe

A DNA probe for isolating a pyrF gene was generated by PCR amplification from *T. versicolor* genomic DNA with degenerate primers. The degenerate primers were constructed on the basis of a comparison with sequences of known pyrF genes. Genes for orotate phosphoribosyltransferase (referred to as pyrF genes or, in another nomenclature, referred to as ura5 genes) were sought in the following gene databases: a) swissprot, b) sptrembl, c) pir, d) embl, e) genbank, f) em_tags, g) gb_tagsEMBL. Ura5, or pyrF, genes of the following organisms were selected for the sequence comparison: Yarrowia lipolytica, Saccharomyces cerevisiae, Escherichia coli, Rhizomucor circinelloides, Colletotrichum graminicola, Trichoderma reesei and Sordaria macrospora. The amino acid sequences of said pyrF genes were compared. It was possible by the

comparison of sequences to identify three peptides with a length of from 6 to 9 amino acids which were completely conserved in all pyrF proteins. Two of these peptides were back-translated into DNA, taking account of degenerate codons, in order to produce degenerate primers. The primers had the following sequences (the abbreviation I refers to the base inosine):

(See FIG. 3)

Primer A: 5'-TTYGGICCIGCITAYAARGGIATHCC-3' SEQ ID NO: 4

Primer B: 5'-TTICCICCYTCICCRTGRTCYTT-3' SEQ ID NO: 5

(See FIG. 4)

PCR amplifications were carried out in accordance with the prior art as stated by the manufacturer (PCR kit from Qiagen, Hilden): a 50 µl PCR reaction contained 100 ng of chromosomal T. versicolor DNA (isolated as described in the 2nd example), the buffer provided by the manufacturer and, in addition, 1.25 U of Taq polymerase, 1.25 mM $MgCl_2$, 0.2 mM of each of the four dNTPs (dATP, dCTP, cGTP, dTTP) and in each case 100 pmol of primers A and B. The other conditions for the specific amplification of the desired PCR product were: 4 min at 94°C, followed by 10 cycles of 1 min at 94°C, 1 min at 45°C and 1 min at 65°C, and 30 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C. A PCR product of about 140 bp was obtained. The PCR product was purified by agarose gel electrophoresis, cloned into the pCR-Script vector (cloning kit from Stratagene, Heidelberg) and transformed into E. coli. The plasmid was isolated from cultivation of transformed E. coli. A DNA sequence analysis from the 5' and 3' ends confirmed that the cloned DNA fragment was the fragment of a pyrF gene

To prepare the DNA probe for screening pyrF genes, the pyrF-specific PCR fragment was cut out by treatment with Not I and Eco RI, isolated by agarose electrophoresis and labeled with the nonradioactive "Gene Images" detection kit from Amersham, Braunschweig.

3.4 kb of sequence information was found from the longest of the pyrF clones. This pyrF clone was called pyrF61 (SEQ ID NO: 1). The pyrF61 clone contained sequence information for the pyrF structural gene (coding region, SEQ ID NO: 1, bp 1133-1877). The coding sequence region additionally contained an intron (SEQ ID NO: 1, bp 1226-1286) which is not translated into amino acid sequence. The corresponding pyrF cDNA gene is indicated in SEQ ID NO: 2. The pyrF structural gene present in the pyrF61 clone, without the intron sequence, codes for a protein having the amino acid sequence indicated in SEQ ID NO: 3.

In addition, the pyrF61 clone also contained sequence information in the region 5' upstream of the ATG start codon (promoter region, SEQ ID NO: 1, bp 1-1132) and sequence information in the region 3' downstream of the stop codon (terminator region, SEQ ID NO: 1, bp 1878-3448). These are novel genetic regulatory elements for Trametes versicolor which can be used for producing expression vectors for gene expression in filamentous fungi from the class Basidiomycetes.

4th example

Functional linkage of the Trametes versicolor GAPDH promoter to the pyrF gene from Trametes versicolor

A: Cloning of the pyrF gene into the pBluescript vector

For further processing, the pyrF gene from pyrF61 was recloned into the pBluescript vector. For this purpose, the pyrF gene was isolated as 1.6 kb Sac I-Spe I fragment from the pyrF61 clone obtained in the 3rd example and was subcloned into the pBluescript vector which had previously been cut with Sac I and Spe I. The 4.6 kb plasmid resulting therefrom was called pPyrF1.

B: Incorporation of a linker into pPyrF1 for functional linkage of the ATG translation start codon of the pyrF gene to the GAPDH promoter

The pPyrF1 vector was cut with Sac I, and the linearized vector 4.6 kb in size was isolated by agarose gel electrophoresis and dephosphorylated by treatment with alkaline phosphatase. The vector prepared in this way was ligated to the linker oligonucleotides PyF-1 (SEQ ID NO: 6) and PyF-2 (SEQ ID NO: 7). PyF-1 and PyF-2 had the following sequence:

(See FIG. 5)

Oligo PyF-1:

5'-CTAGACATGTCGCTCGAAAAATACCAGACAGAGCT-3' / SEQ ID NO: 6

Oligo PyF-2:

5'-CTGTCTGGTATTTTTCGAGCGACATGTCTAGAGCT-3' / SEQ ID NO: 7

(See FIG. 6)

The cleavage site for the restriction endonuclease BspLU11 I, which can be used for functional linkage to the GAPDH promoter from T. versicolor, is underlined in PyF-1 and PyF-2.

Ligation mixtures of Sac I-cut pPyrF1 with the linker oligos PyF-1 and PyF-2 were transformed into E. coli Top 10F' cells. Positive clones contained a newly introduced BspLU11 I cleavage site (in addition to two previously present in pPyrF1). The correct orientation of the incorporated linker, with which a BspLU11 I cleavage site had been introduced at the start ATG codon of the pyrF gene, was determined by DNA sequence analysis. The vector produced in this way (about 4.5 kb in size) was called pPyrF2.

C: Incorporation of the T. versicolor GAPDH promoter into the pUC19 vector

The DNA sequence of the promoter for the T. versicolor GAPDH gene is disclosed in DE-A-19814853, SEQ ID NO: 3, bp 1-1542. A promoter fragment about 1 kb in size of the GAPDH gene was isolated as Sph I fragment and cloned into a pUC19 vector. Analysis by double digestion with the restriction endonucleases Eco RI (present in the polylinker of pUC19) and BspLU11 I (present in the GAPDH promoter fragment) was followed

Primer A: 5'-TTYGGICCIGCITAYAARGGIATHCC-3' SEQ ID NO: 4

FIG. 3

Primer B: 5'-TTICCICCYTCICCRTGRTCYTT-3' SEQ ID NO: 5

FIG. 4

Oligo PyF-1:
5'-CTAGACATGTCGCTCGAAAAATACCAGACAGAGCT-3' SEQ ID NO: 6

FIG. 5

Oligo PyF-2:
5'-CTGTCTGGTATTTTTCGAGCGACATGTCTAGAGCT-3' SEQ ID NO: 7

FIG. 6

pyrF gene and its use

The invention relates to a pyrF gene and its use as selection marker gene for an expression system for producing proteins in fungi of the genera Trametes, Coriolus or Polyporus.

Various prokaryotic and eukaryotic expression systems are known for producing proteins. The application DE-A-19814853 describes in detail the prior art in this regard. DE-A-19814853 itself discloses a process for transforming filamentous fungi from the genera Trametes and Polyporus, with which it is possible to achieve significantly higher production rates for a protein expressed in each case. The application discloses expression vectors which comprise genetic regulatory elements for expression in filamentous fungi of the class Basidiomycetes. On transformation of filamentous fungi of the class Basidiomycetes they permit positive transformants to be selected on the basis of the complementation of an auxotrophic gene defect.

The gene defect disclosed in DE-A-19814853 relates to the pyrG gene. This gene codes for orotidine-5'-phosphate decarboxylase. DE-A-19814853 also discloses strains with a defect in the pyrG gene which are able to grow on minimal medium only in the presence of uridine (uridine auxotrophy). After transformation of these strains with DNA vectors which comprise an intact pyrG gene, the uridine-auxotrophic strains again grow on minimal medium without uridine (uridine prototrophy).

Uridine-auxotrophic strains are isolated in the state of the art (Boeke et al., Methods Enzymol. (1987) 154, 164-175) by treatment with the genotoxic substance 5-fluoroorotic acid (FOA). Uridine-auxotrophic strains generated on treatment with FOA have a genetic defect either in the pyrG gene or in the pyrF gene. The pyrF

gene is also called the ura5 gene. It codes for the enzyme orotate phosphoribosyltransferase.

Uridine-auxotrophic Basidiomycetes strains with a defect in the pyrF gene would also be valuable strains for transformation with the aim of producing proteins if the intact pyrF gene from Basidiomycetes were available as selection marker gene for efficient transformation. However, pyrF genes have to date been described only for fungi from the class Ascomycetes such as, for example, Podospora anserina (Gene 53 (1987), 201-209), Kluyveromyces lactis (unpublished, the DNA sequence is deposited in the "Genbank" database under the accession number klj001358.gb_pl) or Yarrowia lipolytica (M. Sanchez et al., Yeast 11 (1995), 425-433). On the other hand, no pyrF genes from filamentous fungi from the class Basidiomycetes such as, for example, of the genera Trametes, Coriolus or Polyporus are known.

One object of the present invention is to provide pyrF genes from filamentous fungi from the class Basidiomycetes. These genes are suitable for use as selection marker genes for the transformation of uridine-auxotrophic strains.

The present invention relates to a DNA sequence which codes for a protein having the enzymatic activity of orotate phosphoribosyltransferase (pyrF activity), which comprises the chromosomal DNA sequence SEQ ID NO: 1 from position 1133 up to and including position 1877, or comprises the cDNA sequence SEQ ID NO: 2 from position 1 up to and including position 684, or comprises a DNA sequence having a sequence homology of more than 60% with the DNA sequence SEQ ID NO: 1 or SEQ ID NO: 2.

A preferred DNA sequence has a sequence homology of more than 70% with the DNA sequence SEQ ID NO: 2.

In a particularly preferred embodiment, the present invention comprises a DNA sequence having a sequence homology of more than 80% with the DNA sequence SEQ ID NO: 2.

All the values mentioned for the homology in the present invention relate to results obtained with the computer program "Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, Wisconsin". The homology is determined by searching the database with the subprogram "blast" and the preset values (hit frequency 10.0). The sequences with the greatest similarity are then examined for homology using the subprogram "gap". The preset parameters "gap creation penalty 50" and "gap extension penalty 3" are used in this in order to compare DNA sequences. The preset parameters "gap weight 8" and "length weight 2" are used to compare amino acid sequences.

The DNA sequence of the invention SEQ ID NO: 1 from position 1133 up to and including position 1225 and from position 1287 up to and including position 1877, and the cDNA sequence derived therefrom SEQ ID NO: 2 from position 1 up to and including position 684 codes for a protein having pyrF activity.

The present invention therefore also relates to a protein having pyrF activity, which comprises the amino acid sequence SEQ ID NO: 3 or comprises an amino acid sequence having a sequence homology of more than 60% with amino acid sequence SEQ ID NO: 3.

The preferred amino acid sequence has a sequence homology of more than 70% with the amino acid sequence SEQ ID NO: 3.

A particularly preferred amino acid sequence in the present invention is one with a sequence homology of

more than 80% with the amino acid sequence SEQ ID NO: 3.

The DNA sequence SEQ ID NO: 1 from position 1226 up to and including position 1286 is an intron which is not translated into amino acid sequence.

The DNA sequence SEQ ID NO: 1 represents from position 1 to position 1132 the DNA sequence for the promoter region for transcription of the pyrF gene from Trametes versicolor. This promoter sequence can be replaced by any other promoter sequences for the transcription.

The DNA sequence of the invention can be obtained, for example, by cloning from the Basidiomycetes strain Trametes versicolor TV-1 (deposited at the DSMZ Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, D-38124 Braunschweig under the number DSM 11523). For this purpose, a Trametes versicolor TV-1 gene library is constructed by methods known per se. This may be a cDNA or a genomic gene library.

The DNA sequence of the invention is isolated from the gene library by using DNA probes which contain pyrF-specific DNA sequences. Such DNA probes can be obtained, for example, by a PCR reaction using DNA primers from genomic DNA of Trametes versicolor TV-1.

The primers used are degenerate DNA sections with a length of, preferably, 23 to 26 bp, whose sequence is established by comparison with sequences of known pyrF genes. The DNA sections suitable as primers are preferably obtained by oligonucleotide synthesis of the established DNA sections. A pyrF gene of the invention can be isolated, for example, as described in examples 1 to 3.

A pyrF gene which has been isolated in this way, for

example, can be modified at any desired position in the sequence by techniques known to the skilled worker, such as, for example, site directed mutagenesis. The invention therefore also comprises a DNA sequence coding for a protein having pyrF activity comprising a DNA sequence with a sequence homology of more than 60%, preferably 70%, particularly preferably 80%, with the DNA sequence SEQ ID NO: 2 from position 1 up to and including position 684.

To express the DNA of the invention, the latter is cloned in an expression vector in a manner known per se, and this expression vector containing the pyrF gene is introduced into a microorganism and expressed in the microorganism.

The invention therefore also relates to an expression vector which comprises a pyrF gene of the invention.

The expression vectors of the invention are particularly suitable for expressing genes which code for proteins in a host organism of the genus Trametes, Coriolus and Polyporus. Genes which code for proteins mean for the purpose of the invention also the cDNA genes derived from the structural genes of the proteins. The proteins may be proteins which are heterologous for the host organism or proteins which are homologous for the host organism.

The expression vector of the invention thus preferably also comprises at least one gene which codes for a protein to be expressed.

The expression vector of the invention particularly preferably comprises at least one gene which codes for a hydrolytic enzyme, for example from the group of cellulases, hemicellulases and lipases or from the group of oxidoreductases such as, for example, the lignin peroxidases, manganese peroxidases, laccases,

cellobiose-quinone oxidoreductase or cellobiose oxidase.

The expression vector of the invention particularly preferably comprises a gene for a laccase.

The expression vector of the invention may be a DNA construct which is integrated into the genome of the host organism and replicated together with the latter. Alternatively, the expression vector may be an autonomously replicating DNA construct which is not integrated into the host genome, such as, for example, a plasmid, an artificial chromosome or a comparable extrachromosomal genetic element.

An expression vector of the invention ought preferably also to comprise the following genetic elements:

a promoter which mediates the expression of a protein-encoding gene in the host organism. This ought preferably to be a strong promoter, so that high expression efficiency can be ensured. The promoter is preferably functionally linked to the 5' end of the gene to be expressed. The promoter may originate from the gene to be expressed, or else the promoter of a foreign gene can be used.

Suitable and preferred promoters are selected from the group of promoters active in filamentous fungi of the class Basidiomycetes, such as, for example, the GAPDH promoter from Trametes versicolor, promoters for laccase genes from Trametes versicolor or Polyporus pinsitus, the promoter for the Ornithine trans-carbamoylase gene or the GAPDH gene from Coriolus hirsutus or the GAPDH promoter from Agaricus bisporus.

The GAPDH promoter from Trametes versicolor is particularly preferred. This promoter is disclosed in DE-A-19814853, example 5, and DE-A-19814853, SEQ ID NO:

3, base 1-1542.

The expression vector ought also preferably to comprise signals suitable for the host organism for termination of transcription and, in eukaryotes, additional signals for polyadenylation, which signals are functionally linked to the 3′ end of the gene to be expressed. Such signals for termination of transcription and polyadenylation are shown, for example, in SEQ ID NO: 1, bp 1878-3448.

The transcription terminator used can be the terminator of the protein-encoding gene to be expressed or else the terminator of a foreign gene. The transcription terminator from a laccase gene is preferably used.

Expression of the proteins can take place intracellularly or, in the presence of a signal sequence capable of functioning for the purpose of secretion, also extracellularly.

If secretion of the expressed protein from the cell is desired, the expression vector of the invention preferably comprises a signal sequence capable of functioning 5′ upstream of the protein-encoding gene. It is additionally possible for a so-called secretion carrier, functionally linked to the 5′ end of the protein-encoding gene, to be present in the expression vector of the invention.

The secretion carrier may be the gene for a secreted protein or the fragment of a gene for a secreted protein. The secretion carrier can be functionally linked to the protein to be secreted in such a way that a fusion protein is produced from the secretion carrier and the protein to be secreted. In another embodiment, the linkage of secretion carrier and the protein to be secreted is designed so that the secretion carrier can be separated from the protein to be secreted. This can

be brought about, for example, by inserting a recognition sequence for a protein-cleaving enzyme into the linkage site between the secretion carrier and the protein to be secreted. An example of this which may be mentioned is the lysine-arginine recognition sequence for the so-called KEX2 protease and an example of a secretion carrier is the glucoamylase from Aspergillus niger (Contreras et al., Bio/Technology (1991) 9, 378-381, Broekhuijsen et al., J. of Biotechnology (1993) 31, 135-145).

DNA sequences which are involved other than as transcription terminators at the 3' end of the protein-encoding gene in the expression and secretion of the expressed ~~expression~~ can likewise be present in the DNA vector of the invention. One example thereof is provided by the gene for the laccase from Neurospora crassa, whose 3' end contains the sequence for 13 amino acids which are deleted during secretion of the protein and are no longer present in the mature protein (Germann et al., J. Biol. Chem. (1988) 263, 885-896).

Preparation of the expression vectors of the invention takes place by methods known in the prior art. Various possibilities are explained in the examples. The methods described therein can be applied by the skilled worker to any desired other vectors, resistance genes, regulatory elements and structural genes.

The invention further relates to microorganisms which comprise an expression vector of the invention.

Microorganisms suitable for the expression of an expression vector of the invention are strains of filamentous fungi from the class Basidiomycetes.

Strains from the genera Trametes, Coriolus and Polyporus are particularly suitable.

Particularly preferred host organisms are monokaryotic strains from the genera Trametes, Coriolus and Polyporus.

Host organisms of the species Trametes versicolor are particularly preferred.

The host organism is preferably distinguished by having a genetic defect in metabolism (auxotrophy), on the basis of which the essential metabolite uridine can no longer be synthesized, and the host organism is no longer able to grow on minimal medium without addition of this metabolite.

The expression vectors of the invention permit the selection of positive transformants on the basis of complementation of an auxotrophic gene defect in the host organism on transformation of fungi selected from the genera Trametes, Coriolus and Polyporus.

The expression vectors of the invention are suitable for producing fungal strains which are capable of efficient expression and secretion of proteins.

The invention therefore also relates to processes for the production of fungal strains which are capable of efficient expression and secretion of proteins.

This process, in which a fungus with an auxotrophic gene defect is transformed as host strain in a transformation mixture, using process steps known per se, with an expression vector which has a gene for complementation of the auxotrophic gene defect in the host strain, and clones transformed with the expression vector are selected from the transformation mixture by selection for complementation of the auxotrophic gene defect, where expression of the gene for complementation of the auxotrophic gene defect in the host strain is controlled by a genetic regulatory

element which is active in the host strain, comprises employing as host strain a uridine-auxotrophic fungus selected from the genera Trametes, Coriolus and Polyporus with a gene defect in the pyrF gene.

The preferred host for gene expression is a monokaryotic basidiomycete from the genus Trametes, Coriolus or Polyporus.

A host particularly preferred for gene expression is of the species Trametes versicolor having a defect in the pyrF gene and being auxotrophic for uridine.

The invention also relates to an expression system comprising a host strain selected from the genera Trametes, Coriolus and Polyporus having a genetic defect in metabolism, on the basis of which the metabolite uridine which is essential for growth is no longer synthesized, and the host strain is no longer able to grow on minimal media without addition of this metabolite, and to an expression vector comprising a selection marker gene which complements the auxotrophic gene defect of the host strain, wherein the host strain has as genetic defect in metabolism a defect in the pyrF gene, and the selection marker gene is a pyrF gene.

The pyrF gene is preferably derived from a fungus of the genus Agaricus, Coriolus, Polyporus, Pleurotus, Phanerochaete, Schizophyllum or Trametes.

Particularly suitable as selection marker gene for the expression system of the invention is the orotate phosphoribosyltransferase gene (pyrF gene) from a filamentous fungus of the class Basidiomycetes Trametes versicolor.

The expression vectors of the invention are particularly suitable for expressing the pyrF gene.

Expression of the pyrF gene from the basidiomycete Trametes versicolor is preferably regulated by the promoter and, where appropriate, terminator for the pyrF gene from Trametes versicolor.

The expression system of the invention is particularly suitable for expressing a gene which codes for a hydrolytic enzyme, for example from the group of proteases, cellulases, hemicellulases and lipases or from the group of oxidoreductases such as, for example, the lignin peroxidases, manganese peroxidases, laccases, cellobiose-quinone oxidoreductase or cellobiose oxidase.

It is particularly suitable and preferred for expressing a gene for a laccase.

Transformation of the host strain takes place by methods corresponding to the prior art. These methods include transformation of protoplasts by the $CaCl_2$/PEG method, transformation by electroporation or biolistic transformation by bombardment with DNA-containing microprojectiles. These methods are described in standard text books.

For example, the gene to be transformed is cloned in a known manner into an expression vector of the invention and introduced by the methods mentioned into a filamentous fungus selected from the genera Trametes, Coriolus and Polyporus.

The gene to be transformed may, however, also be cloned into an expression vector without a selection marker gene and be used together with the vector which complements the auxotrophic gene defect in the host strain for generating transformants (cotransformation).

The strain to be used for the transformation is a

uridine-auxotrophic filamentous fungus selected from the genera Trametes, Coriolus and Polyporus. The relevant strain from the class Basidiomycetes may be a monokaryotic or else a dikaryotic strain. In a preferred embodiment, it is a uridine-auxotrophic strain which has a defect in the pyrF gene.

Particularly preferred for the transformation is a monokaryotic, uridine-auxotrophic, pyrF-deficient strain from the species Trametes versicolor.

The selection of positive transformants takes place, for example, by placing protoplasts, after transformation with vector DNA, on a medium to which is added, for osmotic stabilization of the protoplasts, an addition such as, for example, sorbitol, mannitol or sucrose and which allows the selection of transformants with the complementing pyrF gene.

In a preferred embodiment of the invention, the filamentous fungus Trametes versicolor is transformed in a homologous system with the gene of a laccase from Trametes versicolor. This achieves an increase in the expression rate for said laccase, which significantly improves the production rate in the fermentation of 0.1 g of laccase/l of culture medium which can be achieved in the prior art.

Preferably used for this purpose is the promoter which is intrinsic to the laccase gene or the promoter for a strongly expressed gene from Trametes versicolor. The promoters of the laccase genes I and III, whose isolation and use is described in DE-A-19814853, are preferably used. The promoter of another strongly expressed gene is represented by the GAPDH promoter for the glyceraldehyde-3-phosphate dehydrogenase from Trametes versicolor.

Selection media preferably used are those on which only

Trametes versicolor transformants which have been transformed with a functionally expressed selection marker gene for the pyrF gene are able to grow. Preference is given to the minimal medium described in the 6th example in the absence of uridine, on which pyrF-auxotrophic strains of Trametes versicolor are no longer able to grow or are able to grow again only after addition of uridine.

Successful use of an expression vector of the invention comprising the pyrF gene as selection system depends on efficient expression of the selection marker gene in Trametes transformants. Appropriate expression signals are necessary for efficient expression.

Expression signals from Basidiomycetes bring about functional expression in Trametes versicolor with, surprisingly, considerably greater efficiency than the expression signals otherwise available from Ascomycetes. For this reason, the pyrF selection marker gene in the DNA vectors of the invention is preferably under the control of genetic regulatory elements from Basidiomycetes, particularly preferably from those selected from the genera Trametes, Coriolus and Polyporus.

The pyrF gene is preferably under the control of the 5′ promoter region upstream of it, and the 3′ terminator region downstream of it. A DNA fragment in which the pyrF gene from Trametes versicolor is under the control of the expression signals of the pyrF gene from Trametes versicolor is described in SEQ ID NO: 1.

The pyrF gene may also be under the control of expression signals from Basidiomycetes which differ from those of the pyrF gene. Expression signals which comply with this function include GAPDH promoters of filamentous fungi from the class Basidiomycetes such

as, for example, Coriolus hirsutus, Phanerochaete chrysosporium, Agaricus bisporus or Trametes versicolor, the OCT promoter from Coriolus hirsutus, the promoter of laccase I or of laccase III from Trametes versicolor and the terminator of the GAPDH gene from Agaricus bisporus or the terminators of the laccase I or laccase III gene from Trametes versicolor.

A particularly preferred vector is one in which the pyrF gene from Trametes versicolor is under the control of the expression signals of the GAPDH gene from Trametes versicolor. Such a vector is described in the 4th example.

A particularly preferred vector is one in which the pyrF gene from Trametes versicolor is under the control of the expression signals of the pyrF gene from Trametes versicolor. Such a vector is described in the 3rd example.

The pyrF gene can be any gene which codes for a protein having the enzymatic activity of an orotate phosphoribosyltransferase.

The pyrF gene is preferably derived from a filamentous fungus from the class Basidiomycetes such as, for example, Agaricus bisporus, Phanerochaete chrysosporium, Coriolus hirsutus, Polyporus pinsitus, Schizophyllum commune or Trametes versicolor.

The pyrF gene from Trametes versicolor is particularly preferred.

The invention also relates to a process for producing a protein which comprises employing the expression system of the invention comprising a gene encoding the protein in a manner known per se for protein production, or comprises cultivating in a manner known per se a fungal strain which comprises a gene encoding the protein and

which has been produced by the process of the invention.

Such production processes are known in principle, for example from Eggert et al., Appl. Environ. Microbiol (1996) 62, 1151-1158, Martinez et al., Appl. Microbiol. Biotechnol. (1994) 41, 500-504, or WO 93/08272.

The following examples serve to illustrate the invention further. The standard methods used in the examples for treating DNA or RNA, such as treatment with restriction endonucleases, DNA polymerases, reverse transcriptase etc., and the standard methods such as transformation of bacteria, Southern and Northern analysis, DNA sequencing, radiolabeling, screening and PCR technology were, unless indicated otherwise, carried out as recommended by the manufacturer or, if no manufacturer's instructions were available, in accordance with the prior art known from standard textbooks.

1st example

Isolation of a pyrF-specific DNA probe

A DNA probe for isolating a pyrF gene was generated by PCR amplification from *T. versicolor* genomic DNA with degenerate primers. The degenerate primers were constructed on the basis of a comparison with sequences of ~~kn~~own pyrF genes. Genes for orotate phosphoribosyltransferase (referred to as pyrF genes or, in another nomenclature, referred to as ura5 genes) were sought in the following gene databases: a) swissprot, b) sptrembl, c) pir, d) embl, e) genbank, f) em_tags, g) gb_tagsEMBL. Ura5, or pyrF, genes of the following organisms were selected for the sequence comparison: Yarrowia lipolytica, Saccharomyces cerevisiae, Escherichia coli, Rhizomucor circinelloides, Colletotrichum graminicola, Trichoderma reesei and Sordaria macrospora. The amino acid sequences of said pyrF genes were compared. It was possible by the

comparison of sequences to identify three peptides with a length of from 6 to 9 amino acids which were completely conserved in all pyrF proteins. Two of these peptides were back-translated into DNA, taking account of degenerate codons, in order to produce degenerate primers. The primers had the following sequences (the abbreviation I refers to the base inosine):

Primer A: 5'-TTYGGICCIGCITAYAARGGIATHCC-3' SEQ ID NO: 4
Primer B: 5'-TTICCICCYTCICCRTGRTCYTT-3' SEQ ID NO: 5

PCR amplifications were carried out in accordance with the prior art as stated by the manufacturer (PCR kit from Qiagen, Hilden): a 50 µl PCR reaction contained 100 ng of chromosomal T. versicolor DNA (isolated as described in the 2nd example), the buffer provided by the manufacturer and, in addition, 1.25 U of Taq polymerase, 1.25 mM $MgCl_2$, 0.2 mM of each of the four dNTPs (dATP, dCTP, cGTP, dTTP) and in each case 100 pmol of primers A and B. The other conditions for the specific amplification of the desired PCR product were: 4 min at 94°C, followed by 10 cycles of 1 min at 94°C, 1 min at 45°C and 1 min at 65°C, and 30 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C. A PCR product of about 140 bp was obtained. The PCR product was purified by agarose gel electrophoresis, cloned into the pCR-Script vector (cloning kit from Stratagene, Heidelberg) and transformed into E. coli. The plasmid was isolated from cultivation of transformed E. coli. A DNA sequence analysis from the 5' and 3' ends confirmed that the cloned DNA fragment was the fragment of a pyrF gene

To prepare the DNA probe for screening pyrF genes, the pyrF-specific PCR fragment was cut out by treatment with Not I and Eco RI, isolated by agarose electrophoresis and labeled with the nonradioactive "Gene Images" detection kit from Amersham, Braunschweig.

2nd example

Production of a chromosomal gene library from Trametes versicolor

The strain Trametes versicolor TV-1 (deposited at the DSMZ Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, D-38124 Braunschweig under the number DSM 11523) was used. Mycelium from Trametes versicolor was firstly obtained by cultivation on malt-agar plates (3% malt extract, 0.3% peptone from soybean meal, 1.5% agar-agar, pH 5.0) at 28°C for 7 days. Three pieces were cut out of the malt-agar plates and used to inoculate 100 ml of sterile malt extract medium (3% malt extract, 0.3% peptone from soybean meal, pH 5.0) in 500 ml Erlenmeyer flasks. The culture was incubated at 28°C with shaking at 100 rpm for 7 days. The mycelium suspension produced in this way was filtered with suction through a porcelain funnel and washed with 0.9% saline. 1 g of mycelium from T. versicolor was ground to a fine powder with a mortar and pestle in the presence of liquid nitrogen. The powder was put into a sterile sample vessel and immediately mixed with 5 ml of extraction solution (0.1M Tris-HCl, pH 8.0, 0.1M EDTA, 0.25M NaCl, 0.6 mg/ml proteinase K) and 0.5 ml of a 10% (w/v) sodium lauroylsarcosine solution. After incubation at 50°C for at least 2 h, the mixture was mixed with 0.85 ml of 5M NaCl and 0.7 ml of a 10% (w/v) CTAB solution in 0.7M NaCl and incubated at 65°C for 30 min. After addition of 7 ml of a chloroform/isoamyl alcohol mixture (24:1), the mixture was shaken, the two phases were separated by centrifugation, the aqueous phase was removed, and chromosomal DNA was precipitated by adding 0.6 parts by volume of isopropanol. Further purification of the precipitated DNA took place subsequently on a column (Qiagen Genomic Tip). It was possible in this way to isolate 0.5 mg of chromosomal DNA from 16 g of mycelium.

To produce the chromosomal gene library, 90 µg of

chromosomal DNA from Trametes versicolor TV-1 were cut with Sau 3A in a partial digestion and fractionated by agarose gel electrophoresis. The chromosomal DNA fragments were isolated in the range of sizes of 5-20 kb and greater than 20 kb and in each case cloned into lambda phages which had previously been cut with Bam HI ("Lambda Zap® Express" cloning system from Stratagene). 4×10^4 phages/µg of vector DNA were obtained from the 5-20 kb DNA fraction, and 5×10^4 phages/µg of vector DNA were obtained from the DNA fraction greater than 20 kb. The phages were amplified by infecting the E. coli strain XL-1 Blue MRF'.

3rd example

Isolation of the pyrF gene

The chromosomal gene library from Trametes versicolor TV-1 described in the 2nd example was used. Screening for the genomic pyrF gene was carried out in accordance with the prior art. In a first round of screening, cells of E. coli XL-1 Blue MRF' were initially cultivated on 10 Petri dishes and then infected with 50 000 phages of the chromosomal gene library (5-20 kb fraction, see 2nd example) per Petri dish. After incubation at 37°C, overnight, the newly formed phages were transferred to nylon filters (Stratagene). The filters were then hybridized in accordance with the manufacturer's recommendations with the nonradiolabeled pyrF-specific probe (see 1st example). The hybridization temperature was 60°C. Positive clones were picked and purified by repeating the screening method. After three rounds of isolation, the strongly hybridizing phage clones were isolated in the screening and were recloned into the pBK CMV vector (Stratagene) by "*in vivo* excision" in accordance with the manufacturer's protocol (Stratagene). Analysis of the clones by digestion with restriction endonucleases and PCR showed that all the clones comprised pyrF genes. After analysis of the sequences of three clones, about

3.4 kb of sequence information was found from the longest of the pyrF clones. This pyrF clone was called pyrF61 (SEQ ID NO: 1). The pyrF61 clone contained sequence information for the pyrF structural gene (coding region, SEQ ID NO: 1, bp 1133-1877). The coding sequence region additionally contained an intron (SEQ ID NO: 1, bp 1286-1286) which is not translated into amino acid sequence. The corresponding pyrF cDNA gene is indicated in SEQ ID NO: 2. The pyrF structural gene present in the pyrF61 clone, without the intron sequence, codes for a protein having the amino acid sequence indicated in SEQ ID NO: 3.

In addition, the pyrF61 clone also contained sequence information in the region 5' upstream of the ATG start codon (promoter region, SEQ ID NO: 1, bp 1-1132) and sequence information in the region 3' downstream of the stop codon (terminator region, SEQ ID NO: 1, bp 1878-3448). These are novel genetic regulatory elements for Trametes versicolor which can be used for producing expression vectors for gene expression in filamentous fungi from the class Basidiomycetes.

4th example

Functional linkage of the Trametes versicolor GAPDH promoter to the pyrF gene from Trametes versicolor

A: Cloning of the pyrF gene into the pBluescript vector

For further processing, the pyrF gene from pyrF61 was recloned into the pBluescript vector. For this purpose, the pyrF gene was isolated as 1.6 kb Sac I-Spe I fragment from the pyrF61 clone obtained in the 3rd example and was subcloned into the pBluescript vector which had previously been cut with Sac I and Spe I. The 4.6 kb plasmid resulting therefrom was called pPyrF1.

B: Incorporation of a linker into pPyrF1 for functional linkage of the ATG translation start codon of the pyrF gene to the GAPDH promoter

The pPyrF1 vector was cut with Sac I, and the linearized vector 4.6 kb in size was isolated by agarose gel electrophoresis and dephosphorylated by treatment with alkaline phosphatase. The vector prepared in this way was ligated to the linker oligonucleotides PyF-1 (SEQ ID NO: 6) and PyF-2 (SEQ ID NO: 7). PyF-1 and PyF-2 had the following sequence:

Oligo PyF-1:
5'-CTAGACATGTCGCTCGAAAAATACCAGACAGAGCT-3' SEQ ID NO: 6
Oligo PyF-2:
5'-CTGTCTGGTATTTTTCGAGCGACATGTCTAGAGCT-3' SEQ ID NO: 7

The cleavage site for the restriction endonuclease BspLU11 I, which can be used for functional linkage to the GAPDH promoter from T. versicolor, is underlined in PyF-1 and PyF-2.

Ligation mixtures of Sac I-cut pPyrF1 with the linker oligos PyF-1 and PyF-2 were transformed into E. coli Top 10F' cells. Positive clones contained a newly introduced BspLU11 I cleavage site (in addition to two previously present in pPyrF1). The correct orientation of the incorporated linker, with which a BspLU11 I cleavage site had been introduced at the start ATG codon of the pyrF gene, was determined by DNA sequence analysis. The vector produced in this way (about 4.5 kb in size) was called pPyrF2.

C: Incorporation of the T. versicolor GAPDH promoter into the pUC19 vector

The DNA sequence of the promoter for the T. versicolor GAPDH gene is disclosed in DE-A-19814853, SEQ ID NO: 3, bp 1-1542. A promoter fragment about 1 kb in size of the GAPDH gene was isolated as Sph I fragment and cloned into a pUC19 vector. Analysis by double digestion with the restriction endonucleases Eco RI (present in the polylinker of pUC19) and BspLU11 I (present in the GAPDH promoter fragment) was followed

by selection of a clone in which the BspLU11 I cleavage site was adjacent to the Eco RI cleavage site. The vector 3.7 kb in size produced in this way was called pTVgap (fig. 1).

A unique BspLU11 I cleavage site, which would have interfered with the subsequent vector construction, had previously been deleted from the pUC19 vector used to produce pTVgap. This took place by cutting the pUC19 vector with BspLU11 I and treating the vector, which had been linearized in this way, with Klenow DNA polymerase. The ends of the pUC19 vector, which were offset after the BspLU11 I digestion, were filled in thereby. Subsequent ligation and transformation of E. coli Top 10F' afforded clones which contained a modified pUC19 vector without BspLU11 I cleavage site.

D: Functional linkage of the GAPDH promoter to the pyrF gene

The vector pTVgap was cut with BspLU11 I and Eco RI, and the vector fragment 3.7 kb in size resulting therefrom was isolated by agarose gel electrophoresis and dephosphorylated by treatment with alkaline phosphatase.

The pyrF gene was isolated as BspLU11 I-Eco RI fragment 1.6 kb in size from the pPyrF2 vector. For this purpose, pPyrF2 was first partially cut with BspLU11 I and the linearized vector fragment 4.6 kb in size was isolated by agarose gel electrophoresis. The isolated 4.6 kb fragment was then cut with Eco RI. This resulted in the desired 1.6 kb pyrF gene fragment, which was isolated by agarose gel electrophoresis.

The BspLU11 I-Eco RI vector fragment 3.7 kb in size from pTVgap and the BspLU11 I-Eco RI fragment 1.6 kb in size from pPyrF2 were ligated, and E. coli Top 10F' cells were transformed with the ligation mixture. Clones in which the pyrF gene had been functionally

linked via the BspLU11 I cleavage site to the GAPDH promoter were identified by restriction analysis. Correct linkage of the GAPDH promoter to the start ATG codon of the pyrF gene was confirmed by DNA sequencing. The correct clone had a size of 5.3 kb and was called pPyrFgap (fig. 2).

5th example

Production of Trametes protoplasts and regeneration of fungal colonies

The dikaryotic strains Trametes versicolor TV-1, Trametes versicolor 38070 (obtainable from the American Type Culture Collection, Rockville, MD 20852 USA) and the monokaryotic strain Trametes versicolor F2 100 (deposited at the DSMZ Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, D-38124 Braunschweig under the number DSM 11972) were used to obtain protoplasts. Mycelium from Trametes versicolor was first obtained by cultivation on malt-agar plates (3% malt extract, 0.3% peptone from soybean meal, 1.5% agar-agar, pH 5.0) at 28°C for 7 days. Three pieces were cut out of the malt-agar plates and used to inoculate 100 ml of sterile malt extract medium (3% malt extract, 0.3% peptone from soybean meal, pH 5.0) in 500 ml Erlenmeyer flasks, or 125 ml of the sterile medium in 162 cm^2 cell culture vessels. The culture was incubated at 28°C without shaking for 7 days until a tight mat of mycelium had grown in the culture liquid. The culture liquid was decanted off and fresh medium was added (30 ml for the mycelium in a 100 ml culture). The mycelium was homogenized with an Ultra Turrax (9 500 rpm, 4 min) and incubated at 28°C while shaking at 100 rpm for a further 18 h.

The mycelial suspension produced in this way was harvested by centrifugation at 1 500 rpm (2 000 x g) for 5 min and the mycelium obtained in this way was washed three times by suspending in 0.1M $MgSO_4$, 0.6M sucrose, 0.1M phosphate, pH 5.8 (OMT medium) and

subsequently centrifuging. The isolated mycelium was weighed and stored at 4°C until treated with lytic enzyme.

Protoplasts were produced in the following way: mycelium from a flask was suspended in 15 ml of a freshly prepared and sterile-filtered solution of the lytic enzyme mixture Novozym 234 (3 mg/ml, Novo Nordisk, Bagsvaerd, Denmark) in OMT medium in a sterile Erlenmeyer flask. The mycelium resuspended in the enzyme solution was incubated at 30°C on a shaking incubator (Infors) at a low speed (80 rpm) for 1 to 3 h. During the incubation, the formation of the protoplasts was observed under the microscope. Freely moving protoplasts were normally to be seen after 1 h. The end point of the protoplasting was determined by visual inspection under the microscope, and the protoplasts were separated from the remaining mycelium by filtration through glass wool in a glass filter. The glass wool was carefully washed with ice-cold OMT medium. Protoplasts were isolated by centrifuging the suspension in a sterile sample vessel (2 000 rpm; 2 500 × g, 4°C, 10 min). Further processing of the cells took place at 4°C. The protoplast pellet was washed by suspension in OMT medium and was reisolated by centrifugation. The washing step was repeated if required. The concentration of protoplasts was determined in a counting chamber under the microscope. The protoplast suspension was adjusted to a concentration of 1×10^8 protoplasts/ml for experiments on protoplast regeneration or for transformations.

For regeneration experiments, serial dilutions were prepared from the protoplast suspension and plated out on agar plates which contained 1.5% malt extract, 0.1% Trypticase peptone, 2% glucose, 1.5% agar and, for osmotic stabilization, 0.4M mannitol. The proportion of viable cells was determined, and the possibility of regenerating the resulting protoplasts to mycelial

growth was tested, in this way. In the same way, the proportion of osmotically stable cells (e.g. mycelium fragments) was determined on plates without osmotic stabilizer (without mannitol). The colonies obtained after incubation at 28°C for 7 days were counted. The proportion of viable cells from a number of protoplast preparations was about 0.5%. These results show that viable and regenerable protoplasts can be produced from Trametes versicolor for transformation experiments.

6th example

Isolation of uridine-auxotrophic mutants of Trametes versicolor

Uridine-auxotrophic mutants of Trametes versicolor with a gene defect in pyrimidine metabolism (pyr mutants) were isolated by a method based on that described by Boeke et al., Methods Enzymol. (1987) 154, 164-175. The selective agent used was the genotoxic substance 5-fluoroorotic acid (FOA). Mutagenesis of Trametes versicolor protoplasts took place by UV treatment.

A: UV mutagenesis:

The monokaryotic strain Trametes versicolor F2 100 described in the 5th example was used for the mutagenesis. Protoplasts of this strain were produced as described in the 5th example.

The UV light source used for the mutagenesis was a BioRad UV linker (BioRad, Munich, power 5.8 W/cm^2, distance from the UV source 16 cm). The number of protoplasts used for the mutagenesis was 8×10^9. Protoplasts of Trametes versicolor were placed in a Petri dish and irradiated with UV light for various lengths of time. It emerged from this that, under the described conditions, irradiation for 60 sec was optimal for the subsequent selection of auxotrophic mutants.

B: Selection of uridine-auxotrophic mutants:

The following minimal medium (MM) was used for the selection of uridine-auxotrophic mutants:

Glucose	20	g/l
Agar	15	g/l
Potassium dihydrogen phosphate	1	g/l
Magnesium sulfate	0.5	g/l
Disodium hydrogen phosphate	0.1	g/l
Adenine	27.5	mg/l
DL-Phenylalanine	0.15	g/l
L-Asparagine	2.5	g/l
Thiamine	0.48	mg/l
Calcium chloride	10	mg/l
Iron sulfate	10	mg/l
Copper sulfate	2	mg/l
Zinc sulfate	1	mg/l
Manganese sulfate	1	mg/l

pH 5.0, adjusted with sulfuric acid.

The MM was supplemented with 0.6M sucrose (MMS) for the osmotic stabilization of protoplasts. For liquid cultures, the MM was prepared without agar.

Initially, the MMS was supplemented with various concentrations of FOA and 10 mM uridine in order to characterize the host properties on selective medium for various Trametes strains. It emerged that MMS with 1.5 mg/ml FOA and 10 mM uridine (selective MMS) completely suppressed growth of the Trametes strains investigated.

Plates with selective MMS were inoculated with UV-mutagenized protoplasts (described in section A) and incubated at 28°C for 21 days. In contrast to unmutagenized protoplasts, growth of 35 colonies was observed. These potential pyr-deficient mutants were, in order to characterize the uridine-auxotrophic phenotype in detail, placed on MM plates, MM plates with 10 mM uridine and selective MM plates, and the

growth was compared with the F2 100 initial strain. In this, 13 of the 35 picked colonies of Trametes mutants unambiguously showed a pyr-deficient phenotype. This is depicted by way of example in table 1 for the wild-type strain and three mutants.

Table 1
Growth of Trametes versicolor mutants on various minimal media

Strain	MM	MM + 10 mM uridine	MM + 10 mM uridine + 1.5 mg/ml FOA
F2 100	+	+	-
F2 100C2-1	-	+	+
F2 100C2-8	-	+	+
F2 100C4-13	-	+	+

C: Identification of pyrF mutants

Mutagenesis with FOA may lead either to mutants in the desired pyrF gene (orotate phosphoribosyltransferase) or in the pyrG gene (orotidine-5'-phosphate decarboxylase). Differentiation of pyrG mutants and pyrF mutants took place by transformation with the pyrF gene from Trametes versicolor, isolation of which was described in the 3rd example (plasmid pyrF61). In parallel with this, uridine-auxotrophic T. versicolor strains were also transformed with the plasmid pPyrFgap (see 4th example for preparation). Transformation of Trametes versicolor is described in the 7th example.

With 6 of the 13 isolated pyr-deficient mutants it was possible to observe colonies on MM after transformation with the plasmids pyrF61 and pPyrFgap. This indicates that these six mutants were deficient in the pyrF gene. The three strains F2 100C2-1, F2 100C2-8 and F2 100C4-13 could be transformed repeatedly with the highest frequency and were used for the subsequent investigations. Comparison of the plasmids pyrF61 and pPyrFgap in relation to transformation frequency showed

no significant differences, so that the pyrF promoter was sufficient for isolating transformants.

The pyrF gene described in the 2nd example is a novel selection marker gene for the transformation of Trametes versicolor. The strains Trametes versicolor F2 100C2-1, F2 100C2-8 and F2 100C4-13 are the first pyrF-deficient strains of Trametes versicolor to be described to date. These pyrF-deficient strains can be used as host organisms for the transformation of Trametes versicolor and are thus novel and valuable host organisms for protein expression and protein excretion in filamentous fungi from the class Basidiomycetes. The use of the strain F2 100C2-1 for this purpose is described in the following examples.

7th example
Transformation of pyrF-deficient Trametes versicolor strains with the pyrF gene from Trametes versicolor

A: Isolation of transformants

Protoplasts of T. versicolor F2 100C2-1 were produced by the method described in the 5th example. In this case, the culture medium for the auxotrophic strain was supplemented with 10 mM uridine. Transformation was carried out with the vector pyrF61 (described in the 3rd example) or pPyrFgap (described in the 4th example).

Protoplasts were produced from Trametes versicolor F2 100C2-1 as described in the 5th example and were suspended in a final concentration of 10^8/ml. 0.1 ml aliquots of the protoplasts were mixed in each case with 10 µg of DNA of the relevant plasmid in incubation vessels with a volume of 12 ml and incubated on ice for 30 min. After this, 1.25 ml of a PEG4000 solution was added slowly and with repeated mixing to the transformation mixture. After incubation at room temperature for a further 20 min, the reaction vessels

were filled with the OMT medium described in the 5th example, mixed and centrifuged at 2 000 × g and 4°C for 10 min. The pellets were resuspended and plated out on osmotically stabilized MMS plates without uridine (described in the 6th example). The plates were incubated at 28°C for 14 days and checked for growth of colonies. Transformation rates of 0.5-3 transformants/µg of plasmid DNA were achieved in various experiments.

B: Purification of the transformants

Mycelium of the resulting transformants was picked and purified by plating out on fresh MM plates. The inoculum was applied as a spot in the middle of the plate in this case. After incubation at 28°C for about 7 days, radial mycelial growth was observable. This purification process was repeated, taking the mycelium for the inoculum from the edge of the first purification plate. MM plates were then reinoculated with inoculum from the second purification plate and incubated at 28°C until the plates were completely covered with mycelial growth.

C: Analysis of the transformants

Transformants of Trametes versicolor were investigated by Southern blot analysis for integration of the plasmid pyrF61. This was done by producing mycelium of various transformants and, as a control, the pyrF-deficient strain F2 100C2-1 in liquid culture (see 2nd example, malt extract medium, with addition of 10 mM uridine for F2 100C2-1). Chromosomal DNA was isolated from the isolated mycelium as described in the 2nd example.

3 µg of chromosomal DNA from each of the investigated transformants and the untransformed, uridine-auxotrophic F2 100C2-1 initial strain, and 100 ng of the plasmid pyrF61 were cut with Nco I and then separated by agarose gel electrophoresis and blotted

onto nylon filters (Qiagen). The DNA probe used was Nco I-cut plasmid pyrF61, nonradiolabeled as described in the 1st example. It was possible to detect with this DNA probe both the pyrF gene and the vector portions from the respective plasmid.

The temperature for the hybridization of the DNA blotted onto nylon filters with the unradiolabeled DNA probe was 60°C. Otherwise, the conditions described in the specialist literature for Southern blots were complied with. Southern blots were analyzed by autoradiography. Besides other fragments, it was possible to detect two Nco I fragments which were derived from the pBK CMV vector portion of pyrF61 and had a length of 0.7 kb and 1.9 kb respectively. These two fragments were detectable only in the transformants but not in the uridine-auxotrophic strain F2 100C2-1. This result confirms that on transformation of the uridine-auxotrophic Trametes versicolor strain F2 100C2-1 the plasmid pyrF61 had been integrated into the genome and led to productive expression of the selection marker gene pyrF, whereby the uridine auxotrophy of this strain was complemented.

8th example

Use of the pyrF gene for producing laccase-overproducing Trametes versicolor strains

A. Transformation of T. versicolor

Protoplasts of T. versicolor were produced by the process described in the 5th example. The vector pyrF61 described in the 3rd example, and the laccase expression vector pLac3gap were used for the transformation. The two vectors were used in cotransformation experiments where the selection marker gene and the gene to be expressed were present on separate plasmids. The production of pLac3gap was disclosed in DE-A-19814853, 6th example. In pLac3gap, the gene for laccase III from T. versicolor is functionally linked to the GAPDH promoter from T. versicolor.

Protoplasts of the pyrF-deficient strain Trametes versicolor F2 100C2-1 were produced as described in the 7th example and were suspended in a final concentration of 10^8/ml. 0.1 ml aliquots of the protoplasts were mixed with 10 µg of DNA of each of the plasmids pLac3gap and pyrF61 in incubation vessels with a volume of 12 ml and incubated on ice for 30 min. After this, 1.25 ml of a PEG4000 solution was added slowly and with repeated mixing to the transformation mixture. After incubation at room temperature for a further 20 min, the reaction vessels were filled with the OMT medium described in the 5th example, mixed and centrifuged at 2 000 x g and 4°C for 10 min. The pellets were resuspended and plated out on osmotically stabilized MM without uridine (described in the 6th example). The plates were incubated at 28°C for 14 days and examined for growth of colonies. Transformation rates of 0.5-3 transformants/µg of DNA of the selection marker plasmid pyrF61 were achieved in various experiments.

The resulting transformants were picked and purified twice as described in the 7th example by plating out on fresh plates with MM selection medium without uridine. Selective plates were then inoculated anew with inoculum from the second purification plate and, after the plates were completely covered with mycelial growth, the laccase production was checked in shaken flask cultures.

B: Culturing in a shaken flask

For culturing in a shaken flask, 2 cm^2 of mycelium were punched out of a plate showing full growth and were crushed under sterile conditions and used to inoculate a preculture of 50 ml (in a 250 ml Erlenmeyer flask) of malt extract medium (see 1st example). The preculture was incubated at 28°C while shaking at 120 rpm for 6 days. On the sixth day, the preculture was homogenized with an Ultra Turrax at 9 500 rpm for

30 sec and used to inoculate 250 ml of main culture medium (for composition, see MM in the 6th example) in a 1l Erlenmeyer flask. The main culture was then again incubated at 28°C while shaking at 120 rpm. Laccase production was measured each day starting on the second day of culturing. Laccase activity was measured by photometry using the substrate ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) at 420 nm. (Extinction coefficient of ABTS at 420 nm ε_{420}: $3.6 \times 10^4 [1 \times mol^{-1} \times cm^{-1}]$. In this, 1U of laccase activity corresponded to the conversion of 1 µmol of ABTS/min at 37°C and a pH of 4.5. The maximum laccase production in shaken flask cultures was reached 10-14 days after starting the main culture. Table 2 shows a comparison of various transformants with the untransformed starting strain Trametes versicolor F2 100. For the untransformed strain F2 100, laccase production was additionally determined after induction with the inducer 2,5-xylidine described in the literature (Yaver et al., Applied and Environmental Microbiology (1996) 62, 834-841). As is evident from table 2, laccase production in a shaken flask was increased with the best transformants of the strain F2 100 by a factor of 14 (without induction) and by a factor of 6 (with induction) compared with the untransformed starting strain.

Table 2

Trametes versicolor strain	Maximum laccase production (U/ml)
F2 100	4.60
F2 100/xylidine*	11.20
TV L3F-4	15.20
TV L3F-7	42.50
TV L3F-9	17.60
TV L3F-14	51.50
TV L3F-21	33.90

TV L3F-29	64.80
TV L3F-35	35.10
TV L3F-35	13.80
TV L3F-51	56.70

* Induction took place three days after starting the main culture by adding 2,5-xylidine (final concentration 1.5 mM).

Claims:

1. A DNA sequence which codes for a protein having the enzymatic activity of orotate phosphoribosyl-transferase (pyrF activity), which comprises the DNA sequence SEQ ID NO: 1 from position 1133 up to and including position 1877, or comprises the DNA sequence SEQ ID NO: 2 from position 1 up to and including position 684, or comprises a DNA sequence having a sequence homology of more than 60% with the said regions of the DNA sequence SEQ ID NO: 1 or SEQ ID NO: 2.

2. A protein having pyrF activity, which comprises the amino acid sequence SEQ ID NO: 3 or an amino acid sequence having a sequence homology of more than 60% with the amino acid sequence SEQ ID NO: 3.

3. An expression vector which comprises a DNA sequence as claimed in claim 1.

4. A microorganism which comprises an expression vector as claimed in claim 3.

5. A process for producing fungal strains which are capable of efficient expression and secretion of proteins, in which a fungal strain with an auxotrophic gene defect is transformed as host strain in a transformation mixture, process steps known per se using with an expression vector which has a gene for complementation of the auxotrophic gene defect in the host strain, and clones transformed with the expression vector are selected from the transformation mixture by selection for complementation of the auxotrophic gene defect, where expression of the gene for complementation of the auxotrophic gene defect in

the host strain is controlled by a genetic regulatory element which is active in the host strain, which comprises employing as host strain a uridine-auxotrophic fungus selected from the genera Trametes, Coriolus and Polyporus with a gene defect in the pyrF gene.

6. An expression system comprising a host strain selected from the genera Trametes, Coriolus and Polyporus having a genetic defect in metabolism, on the basis of which the metabolite uridine which is essential for growth is no longer synthesized, and the host strain is no longer able to grow on minimal media without addition of this metabolite, and an expression vector comprising a selection marker gene which complements the auxotrophic gene defect of the host strain, wherein the host strain has as genetic defect in metabolism a defect in the pyrF gene, and the selection marker gene is the pyrF gene from a fungus of the class Basidiomycetes.

7. A process for producing a protein, which comprises employing an expression system as claimed in claim 6 comprising a gene encoding the protein in a manner known per se for protein production, or comprises cultivating a microorganism as claimed in claim 4 comprising a gene encoding the protein or a fungal strain produced by a process as claimed in claim 5 comprising a gene encoding the protein in a manner known per se, and obtaining the protein from the culture.

Fig. 1

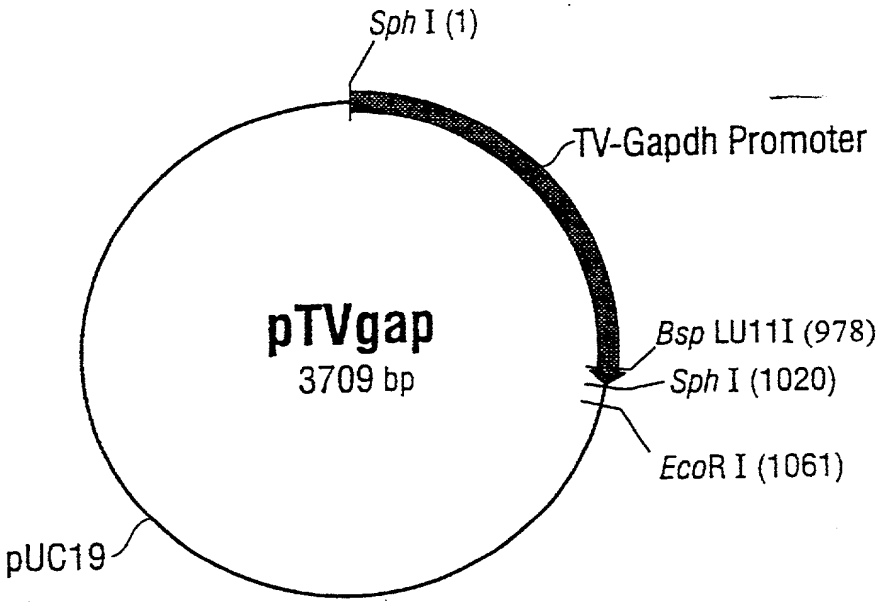
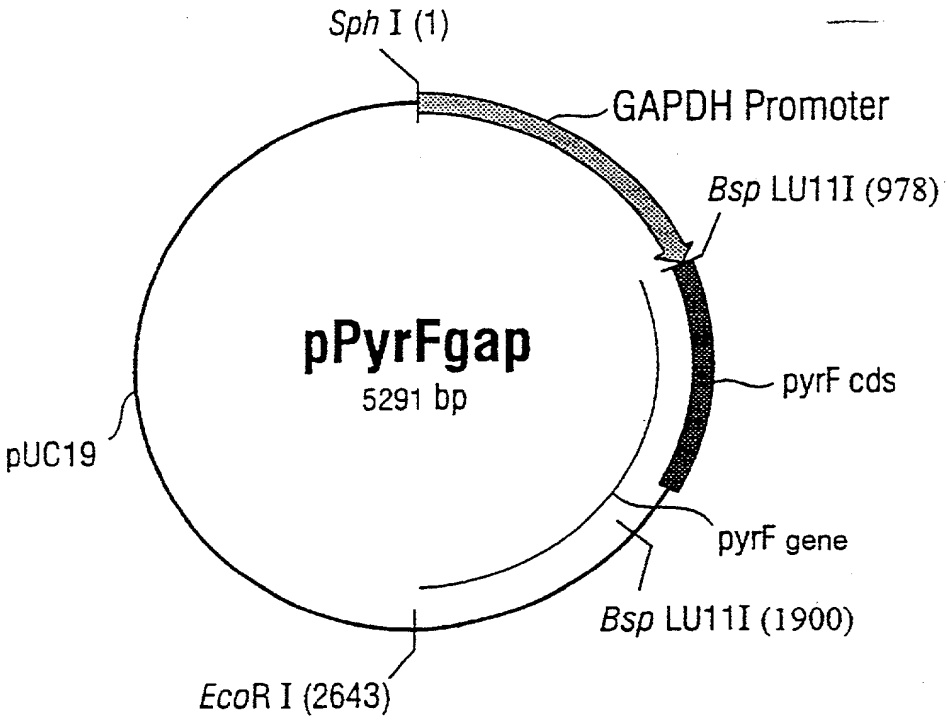


Fig. 2



COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY
(Includes Reference to PCT International Applications)

ATTORNEY'S DOCKET NUMBER
PFALLER-1 (PCT)

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled:

PYRF GENE AND ITS USE

the specification of which (check only one item below):

[ ] is attached hereto.

[ ] was filed as United States application

Serial No. _____

on _____,

and was amended

on _____ (if applicable).

[x] was filed as PCT international application

Number PCT/EP00/06091

on June 29, 2000,

and was amended under PCT Article 19

on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119:

COUNTRY (if PCT, indicate "PCT")	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 USC 119
Germany	199 34 408.6	22 July 1999	[X] YES [ ] NO
			[ ] YES [ ] NO
			[ ] YES [ ] NO
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			[ ] YES [ ] NO

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(Includes Reference to PCT International Applications)

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(Application Number) (Filing Date)

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PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120:

U.S. APPLICATIONS			STATUS (Check One)		
U.S. APPLICATION NUMBER	U.S. FILING DATE		PATENTED	PENDING	ABANDONED
PCT APPLICATIONS DESIGNATING THE U.S.					
PCT APPLICATION NO.	PCT FILING DATE	U.S. SERIAL NUMBERS ASSIGNED (if any)			

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (List name and registration numbers):

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1	POST OFFICE ADDRESS	POST OFFICE ADDRESS NIBELUNGENSTRASSE 6	CITY MÜNCHEN	STATE & ZIP CODE/COUNTRY D-82319/GERMANY

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201	SIGNATURE OF INVENTOR 202	SIGNATURE OF INVENTOR 203
X Rupert Pfaller		
DATE X 02.01.2002	DATE	DATE

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tgggcgggaa gggacgtgtg ccgacgatcc tgaggatgaa ggacctcatg aagtggttgc 1800
aggagcacgg ccggacggag gagcttgcga agatgcaaga gtactgggag cagtacggcg 1860
cgaaggaaag cgaatgagaa gacacgaagg cagttgtgta ctaggtgagt aacaccacgc 1920
tacatcgatc catccactaa acccatgcag atgaagaccc actgtacaat ttctcggtac 1980
ctgtcacgtt gaacgcaaag agccgaagat gtgagagtac acatgccatt catcccgata 2040
tatagcacaa gaacatgtag taatagaacc tgcagaaaca caaagcatga tcagcaagac 2100
tccatgggca ctgagttatg atgaactaac cgctatcacc aaaaacaccg ctcttattcg 2160
cccaaccgac gacccgaacc ccagttatat cctcccacac cgctcgcagc agcagcagca 2220
gcagcctgct ccctgaccct ccgtgggggt acaacatgca cgccccacc gacattcgca 2280
acgcccccga ccccacccgt cgcgcccccca ctagcagact ccccgaacca cccgcgcagc 2340
cacgccggcg ccgtacctgc agccctgagc gagccggtgt caaagacagt ccaccaccag 2400
aggtggccga cgacagcccc agagatgctg atggcagcgg cgccggggcc gcccatgagg 2460
aggtccatgc cgacgagcat ctacggaagg tagatgacgg ggaaggtgat gagcccgaag 2520

aaggatgtct gggacccagg tggggcgagc cggcaggaga cgtaggtgag cgcgaggagg 2580

agcgccgggg tgtgcacaaa ggtgccgagc ggaatgttga ggccctggtg tgaggcgggt 2640

tagcgcaaag gtcagaggcg ggatgatact attggacgta cgaggatagc aagtcctgcg 2700

agcgagagct gccatgcgta gtctgaagag cggcgggggg agtgtgtctc ttctagctca 2760

ttggaatttc gactagtttc aagtgtacgg tcctcagtat catcatgtat tgcaacagtg 2820

tcatacgcac tagagcatcg caaggtcgaa gatgaagttg atccccgagc ctataaagac 2880

aaggtcagca ccgacatggc atgtagttcag acaagattga gtacgcactt cccaagaaga 2940

agctcgtaaa cactctccag atctacatta agacgtgagt atcgcatacc ttctcagtgc 3000

ctgacttatc tttcatccaa ctacagagac agaaacccac ctcaaacttc tgcgtaacga 3060

actccttcac aaagacgacc ttgtatattg gcaagatttg caggagcact ggcaaggtga 3120

cggcgagaga ggaggcgcat agaaatcgag tgactggagg gattttgcga atctcatcca 3180

tgaaagacat cttgaggaga ctggagctga gtagagcgat agaagtacag caggcagagc 3240

agagacgacg gcagaatgtg gggaagaaca agcaggagga ggagtagagt gattttgaag 3300

taatgaaaag tggcgcaacc taatgcaaag tgtatgaggg acatccgtgg acataaagta 3360

ttccgcacct cgggcaagac attcaatctc agtaatgcac ttcactttcg gagttcaact 3420

tcaaactcga ctttgaaact tgagatcc 3448

<210> 2
<211> 684
<212> Dann
<213> Trametes versicolor

<220>
<221> CDS
<222> (1)..(684)

<400> 2

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atg tcg ctc gaa aaa tac cag aca gag ctc atc gag cac ggc atg acc   48
Met Ser Leu Glu Lys Tyr Gln Thr Glu Leu Ile Glu His Gly Met Thr
  1               5                  10                  15

gcc ggt gcg ctc aag ttc ggg acc ttc acc ctc aaa tca ggc cgg acc   96
Ala Gly Ala Leu Lys Phe Gly Thr Phe Thr Leu Lys Ser Gly Arg Thr
             20                  25                  30

tcg ccc tac ttc ttc aac gcc ggc ctg ctc gcg tcc ggg ccc gtg ctc  144
Ser Pro Tyr Phe Phe Asn Ala Gly Leu Leu Ala Ser Gly Pro Val Leu
         35                  40                  45

gac acg ctg tgc tcc gcg tac gcc gcg acg atc gcg cgc gcg ctc aag  192
Asp Thr Leu Cys Ser Ala Tyr Ala Ala Thr Ile Ala Arg Ala Leu Lys
     50                  55                  60
```

gcg tcg ccc ggg ctg ccc gcg ttc gac gtg ctc ttc ggc ccc gcg tac 240
Ala Ser Pro Gly Leu Pro Ala Phe Asp Val Leu Phe Gly Pro Ala Tyr
65 70 75 80

aag ggc atc ccg ttc gcg gcg ggg acc gcg ctg ctg ctg cac cgc gac 288
Lys Gly Ile Pro Phe Ala Ala Gly Thr Ala Leu Leu Leu His Arg Asp
85 90 95

cac ggc atc acc gtc ggg ttc gcg tac gac cgc aag gag gcg aag gat 336
His Gly Ile Thr Val Gly Phe Ala Tyr Asp Arg Lys Glu Ala Lys Asp
100 105 110

cat ggg gag ggc ggg ata ctt gtg ggc gcg ccg gtg agg ggc aag cgc 384
His Gly Glu Gly Gly Ile Leu Val Gly Ala Pro Val Arg Gly Lys Arg
115 120 125

gtg ctg gtg ctg gac gac gtc gcg acg gcg ggc acg gcg atc cgc cag 432
Val Leu Val Leu Asp Asp Val Ala Thr Ala Gly Thr Ala Ile Arg Gln
130 135 140

gcg att gag act gtg acg aag gag ggg ggc gag gtc gtt ggc gcg gtg 480
Ala Ile Glu Thr Val Thr Lys Glu Gly Gly Glu Val Val Gly Ala Val
145 150 155 160

ttg atg ctc gat cgg cag gag gtg ggc aag gag ggg aag agc acg ctt 528
Leu Met Leu Asp Arg Gln Glu Val Gly Lys Glu Gly Lys Ser Thr Leu
165 170 175

gcg gag gtg gag gcg ctg ttg ggc ggg aag gga cgt gtg ccg acg atc 576
Ala Glu Val Glu Ala Leu Leu Gly Gly Lys Gly Arg Val Pro Thr Ile
180 185 190

ctg agg atg aag gac ctc atg aag tgg ttg cag gag cac ggc cgg acg 624
Leu Arg Met Lys Asp Leu Met Lys Trp Leu Gln Glu His Gly Arg Thr
195 200 205

gag gag ctt gcg aag atg caa gag tac tgg gag cag tac ggc gcg aag 672
Glu Glu Leu Ala Lys Met Gln Glu Tyr Trp Glu Gln Tyr Gly Ala Lys
210 215 220

gaa agc gaa tga 684
Glu Ser Glu
225

<210> 3
<211> 227
<212> PRT
<213> Trametes versicolor

<400> 3
Met Ser Leu Glu Lys Tyr Gln Thr Glu Leu Ile Glu His Gly Met Thr
1 5 10 15

Ala Gly Ala Leu Lys Phe Gly Thr Phe Thr Leu Lys Ser Gly Arg Thr
20 25 30

Ser Pro Tyr Phe Phe Asn Ala Gly Leu Leu Ala Ser Gly Pro Val Leu
35 40 45

Asp Thr Leu Cys Ser Ala Tyr Ala Ala Thr Ile Ala Arg Ala Leu Lys
50 55 60

Ala Ser Pro Gly Leu Pro Ala Phe Asp Val Leu Phe Gly Pro Ala Tyr
65 70 75 80

Lys Gly Ile Pro Phe Ala Ala Gly Thr Ala Leu Leu Leu His Arg Asp
85 90 95

His Gly Ile Thr Val Gly Phe Ala Tyr Asp Arg Lys Glu Ala Lys Asp
100 105 110

His Gly Glu Gly Gly Ile Leu Val Gly Ala Pro Val Arg Gly Lys Arg
115 120 125

Val Leu Val Leu Asp Asp Val Ala Thr Ala Gly Thr Ala Ile Arg Gln
130 135 140

Ala Ile Glu Thr Val Thr Lys Glu Gly Gly Glu Val Val Gly Ala Val
145 150 155 160

Leu Met Leu Asp Arg Gln Glu Val Gly Lys Glu Gly Lys Ser Thr Leu
165 170 175

Ala Glu Val Glu Ala Leu Leu Gly Gly Lys Gly Arg Val Pro Thr Ile
180 185 190

Leu Arg Met Lys Asp Leu Met Lys Trp Leu Gln Glu His Gly Arg Thr
195 200 205

Glu Glu Leu Ala Lys Met Gln Glu Tyr Trp Glu Gln Tyr Gly Ala Lys
210 215 220

Glu Ser Glu
225

<210> 4
<211> 26
<212> Dann
<213> Artificial Sequence

<220>
<223> Description of the artificial sequence: Primer A

<220>
<221> primer_bind
<222> (1)..(26)

<220>
<221> primer_bind
<222> (1)..(26)
<223> n = i

<400> 4
ttyggnccng cntayaargc nathcc 26

<210> 5
<211> 23

<212> Dann
<213> Artificial Sequence

<220>
<223> Description of the artificial sequence: Primer B

<220>
<221> primer_bind
<222> Complement((1)..(23))

<220>
<221> primer_bind
<222> Complement((1)..(23))
<223> n = i

<400> 5
ttncenccyt cncertgrtc ytt 23

<210> 6
<211> 35
<212> Dann
<213> Artificial Sequence

<220>
<223> Description of the artificial sequence: PyF-1

<220>
<221> misc_feature
<222> (1)..(35)

<400> 6
ctagacatgt cgctcgaaaa ataccagaca gagct 35

<210> 7
<211> 35
<212> Dann
<213> Artificial Sequence

<220>
<223> Description of the artificial sequence: PyF-2

<220>
<221> misc_feature
<222> Complement((1)..(35))

<400> 7
ctgtctggta ttttcgagc gacatgtcta gagct 35

gctcgtgttc cggcacgccg tatgctcgta aatcaccttt agaaaacttg aataagtgag 780

agaagatacg aaacgtcagt ctgcacctat ggagatatgt aaaaatcgca aaaacatagc 840

gttgacgcta taaaaaagaa aaggacaaaa tgaccaccgc agcggtcgaa cctgcaatct 900

cctgatccct aggtttgaag gttcatcacc tcaattcgta gtcagacgcg atgccatttc 960

gccaggcggc cgttagaaac gaaactacta cgtttaaacc cgggtataac acagcctagt 1020

attccgtgcg ggccgcgccg ccgataagct tgttttcgtg aactgtcttc cccctcctgc 1080

atctcgattc tcgacctcca tcgccgcgac gatcccttcc ttcccactca ccatgtcgct 1140

cgaaaaatac cagacagagc tcatcgagca cggcatgacc gccggtgcgc tcaagttcgg 1200

gaccttcacc ctcaaatcag gccggtccgt cccctcccta ggctgcgcgc cgctctcccc 1260

gtgaacgctc cctcaccccg cgcaggaccc cgccctactt cttcaacgcc ggcctgctcg 1320

cgtccgggcc cgtgctcgac acgctgtgct ccgcgtacgc cgcgacgatc gcgcgcgcgc 1380

tcaaggcgtc gcccgggctg cccgcgttcg acgtgctctt cgggcccgcg tacaagggca 1440

tcccgttcgc ggcggggacc gcgctgctgc tgcaccgcga ccacggcatc accgtcgggt 1500

tcgcgtacga ccgcaaggag gcgaaggatc atggggaggg cgggatactt gtgggcgcgc 1560

cggtgagggg caagcgcgtg ctggtgctgg acgacgtcgc gacggcgggc acggcgatcc 1620

gccaggcgat tgagactgtg acgaaggagg ggggcgaggt cgttggcgcg gtgttgatgc 1680

tcgatcggca ggaggtgggc aaggaggggg agagcacgct tgcggaggtg gaggcgctgt 1740

tgggcgggaa gggacgtgtg ccgacgatcc tgaggatgaa ggacctcatg aagtggttgc 1800

aggagcacgg ccggacggag gagcttgcga agatgcaaga gtactgggag cagtacggcg 1860

cgaaggaaag cgaatgagaa gacacgaagg cagttgtgta ctaggtgagt aacaccacgc 1920

tacatcgatc catccactaa acccatgcag atgaagaccc actgtacaat ttctcggtac 1980

ctgtcacgtt caacgcaaag agccgaagat gtgagagtac acatgccatt catcccgata 2040

tatagcacaa gaacatgtag taatagaacc tgcagaaaca caaagcatga tcagcaagac 2100

tccatgggca ctgagttatg atgaactaac cgctatcacc aaaaacaccg ctcttattcg 2160

cccaaccgac gacccgaacc ccagttatat cctcccacac cgctcgcagc agcagcagca 2220

gcagcctgct ccctgaccct ccgtgggggc acaacatgca cgcccccacc gacattcgca 2280

acgcccccga ccccacccgt cgcgccccca ctagcagact ccccgaacca cccgcgcagc 2340

cacgccggcg ccgtacctgc agccctgagc gagccggtgt caaagacagt ccaccaccag 2400

aggtggccga cgacagcccc agagatgctg atggcagccg cgccggggcc gcccatgagg 2460

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aaggatgtct gggacccagg tggggcgagc cgggaggaga cgtaggtgag cgcgaggagg 2580

agcgccgcgg tgtgcacaaa ggtgccgagg ggaatgttga ggccctggtg tgaggcgggt 2640

tagcgcaaag gtcagaggcg ggatgatact attggacgta cgaggatagc aagtcctgcg 2700

agcgagagct gccatgcgta gtctgaagag cggcgggggg agtgtgtctc ttctagctca 2760

ttggaatttc gactagtttc aagtgtacgg tcctcagtat catcatgtat tgcaacagtg 2820

tcatacgcac tagagcatcg caaggtcgaa gatgaagttg atccccgagc ctataaagac 2880

aaggtcagca ccgacatggc atgtagtcag acaagattga gtacgcactt cccaagaaga 2940

agctcgtaaa cactctccag atctacatta agacgtgagt atcgcatacc ttctcagtgc 3000

ctgacttatc tttcatccaa ctacagagac agaaacccac ctcaaacttc tgcgtaacga 3060

acccttcac aaagacgacc ttgtatattg gcaagatttg caggagcact ggcaaggtga 3120

cggcgagaga ggaggcgcat agaaatcgag tgactggagg gattttgcga atctcatcca 3180

tgaaagacat cttgaggaga ctggaggtga gtagagcgat agaagtacag caggcagagc 3240

agagacgacg gcagaatgtg gggaagaaca agcaggagga ggagtagagt gattttgaag 3300

taatcaaaag tggcgcaacc taatgcaaag tgtatgaggg acatccgtgg acataaagta 3360

ttccgcacct cgggcaagac attcaatctc agtaatgcac ttcactttcg gagttcaact 3420

tcaaactcga ctttgaaact tgagatcc 3448

<210> 2
<211> 684
<212> Dann
<213> Trametes versicolor

<220>
<221> CDS
<222> (1)..(684)

<400> 2

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atg tcg ctc gaa aaa tac cag aca gag ctc atc gag cac ggc atg acc   48
Met Ser Leu Glu Lys Tyr Gln Thr Glu Leu Ile Glu His Gly Met Thr
 1               5                  10                  15

gcc ggt gcg ctc aag ttc ggg acc ttc acc ctc aaa tca ggc cgg acc   96
Ala Gly Ala Leu Lys Phe Gly Thr Phe Thr Leu Lys Ser Gly Arg Thr
            20                  25                  30

tcg ccc tac ttc ttc aac gcc ggc ctg ctc gcg tcc ggg ccc gtg ctc  144
Ser Pro Tyr Phe Phe Asn Ala Gly Leu Leu Ala Ser Gly Pro Val Leu
        35                  40                  45

gac acg ctg tgc tcc gcg tac gcc gcg acg atc gcg cgc gcg ctc aag  192
Asp Thr Leu Cys Ser Ala Tyr Ala Ala Thr Ile Ala Arg Ala Leu Lys
    50                  55                  60
```

```
gcg tcg ccc ggg ctg ccc gcg ttc gac gtg ctc ttc ggg ccc gcg tac   240
Ala Ser Pro Gly Leu Pro Ala Phe Asp Val Leu Phe Gly Pro Ala Tyr
 65                  70                  75                  80

aag ggc atc ccg ttc gcg gcg ggg acc gcg ctg ctg ctg cac cgc gac   288
Lys Gly Ile Pro Phe Ala Ala Gly Thr Ala Leu Leu Leu His Arg Asp
                 85                  90                  95

cac ggc atc acc gtc ggg ttc gcg tac gac cgc aag gag gcg aag gat   336
His Gly Ile Thr Val Gly Phe Ala Tyr Asp Arg Lys Glu Ala Lys Asp
            100                 105                 110

cat ggg gag ggc ggg ata ctt gtg ggc gcg ccg gtg agg ggc aag cgc   384
His Gly Glu Gly Gly Ile Leu Val Gly Ala Pro Val Arg Gly Lys Arg
        115                 120                 125

gtg ctg gtg ctg gac gac gtc gcg acg gcg ggc acg gcg atc cgc cag   432
Val Leu Val Leu Asp Asp Val Ala Thr Ala Gly Thr Ala Ile Arg Gln
    130                 135                 140

gcg att gag act gtg acg aag gag ggg ggc gag gtc gtt ggc gcg gtg   480
Ala Ile Glu Thr Val Thr Lys Glu Gly Gly Glu Val Val Gly Ala Val
145                 150                 155                 160

ttg atg ctc gat cgg cag gag gtg ggc aag gag ggg aag agc acg ctt   528
Leu Met Leu Asp Arg Gln Glu Val Gly Lys Glu Gly Lys Ser Thr Leu
                165                 170                 175

gcg gag gtg gag gcg ctg ttg ggc ggg aag gga cgt gtg ccg acg atc   576
Ala Glu Val Glu Ala Leu Leu Gly Gly Lys Gly Arg Val Pro Thr Ile
            180                 185                 190

ctg agg atg aag gac ctc atg aag tgg ttg cag gag cac ggc cgg acg   624
Leu Arg Met Lys Asp Leu Met Lys Trp Leu Gln Glu His Gly Arg Thr
        195                 200                 205

gag gag ctt gcg aag atg caa gag tac tgg gag cag tac ggc gcg aag   672
Glu Glu Leu Ala Lys Met Gln Glu Tyr Trp Glu Gln Tyr Gly Ala Lys
    210                 215                 220

gaa agc gaa tga                                                  684
Glu Ser Glu
225
```

<210> 3
<211> 227
<212> PRT
<213> Trametes versicolor

<400> 3

```
Met Ser Leu Glu Lys Tyr Gln Thr Glu Leu Ile Glu His Gly Met Thr
 1               5                  10                  15

Ala Gly Ala Leu Lys Phe Gly Thr Phe Thr Leu Lys Ser Gly Arg Thr
            20                  25                  30

Ser Pro Tyr Phe Phe Asn Ala Gly Leu Leu Ala Ser Gly Pro Val Leu
        35                  40                  45
```

Asp Thr Leu Cys Ser Ala Tyr Ala Ala Thr Ile Ala Arg Ala Leu Lys
50 55 60

Ala Ser Pro Gly Leu Pro Ala Phe Asp Val Leu Phe Gly Pro Ala Tyr
65 70 75 80

Lys Gly Ile Pro Phe Ala Ala Gly Thr Ala Leu Leu Leu His Arg Asp
85 90 95

His Gly Ile Thr Val Gly Phe Ala Tyr Asp Arg Lys Glu Ala Lys Asp
100 105 110

His Gly Glu Gly Gly Ile Leu Val Gly Ala Pro Val Arg Gly Lys Arg
115 120 125

Val Leu Val Leu Asp Asp Val Ala Thr Ala Gly Thr Ala Ile Arg Gln
130 135 140

Ala Ile Glu Thr Val Thr Lys Glu Gly Gly Glu Val Val Gly Ala Val
145 150 155 160

Leu Met Leu Asp Arg Gln Glu Val Gly Lys Glu Gly Lys Ser Thr Leu
165 170 175

Ala Glu Val Glu Ala Leu Leu Gly Gly Lys Gly Arg Val Pro Thr Ile
180 185 190

Leu Arg Met Lys Asp Leu Met Lys Trp Leu Gln Glu His Gly Arg Thr
195 200 205

Glu Glu Leu Ala Lys Met Gln Glu Tyr Trp Glu Gln Tyr Gly Ala Lys
210 215 220

Glu Ser Glu
225

<210> 4
<211> 26
<212> Dann
<213> Artificial Sequence

<220>
<223> Description of the artificial sequence: Primer A

<220>
<221> primer_bind
<222> (1)..(26)

<220>
<221> primer_bind
<222> (1)..(26)
<223> n = i

<400> 4
ttyggnccng cntayaargg nathcc 26

<210> 5
<211> 23

<212> Dann
<213> Artificial Sequence

<220>
<223> Description of the artificial sequence: Primer B

<220>
<221> primer_bind
<222> Complement((1)..(23))

<220>
<221> primer_bind
<222> Complement((1)..(23))
<223> n = i

<400> 5
ttnccnccyt cnccrtgrtc ytt 23

<210> 6
<211> 35
<212> Dann
<213> Artificial Sequence

<220>
<223> Description of the artificial sequence: PyF-1

<220>
<221> misc_feature
<222> (1)..(35)

<400> 6
ctagacatgt cgctcgaaaa ataccagaca gagct 35

<210> 7
<211> 35
<212> Dann
<213> Artificial Sequence

<220>
<223> Description of the artificial sequence: PyF-2

<220>
<221> misc_feature
<222> Complement((1)..(35))

<400> 7
ctgtctggta ttttcgagc gacatgtcta gagct 35